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THE ENDOGENOUS METABOLISM OF ANAEROBIC BACTERIA

Final Technical Report

By

E. A. DAWES and P. J. LARGE

March, 1967

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EUROPEAN RESEARCH OFFICE

United States Army

Contract Number DA-91-591-EUC-4044

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SUMMARY

The continuation of an investigation of the endogenous metabolism and survival of non-spore-forming anaerobic bacteria under conditions of starvation is reported. The two glucose-fermenting organisms of the genus Zymomonas, Zymomonas anaerobia and Zymomonas mobilis, differ in that RNA is lost very much more rapidly from Z. mobilis than from Z. anaerobia, although the survival patterns are very similar. Both organisms are resistant to prolonged starvation. Magnesium ions suppress RNA degradation but do not confer protection against death. The remarkably constant protein content of Z. anaerobia does not seem to mask any kind of turnover or preferential degradation and resynthesis of specific proteins. The protein of Peptococcus prévotii is also remarkably stable and the observed release of ammonia is probably derived from deamination of nucleotide bases. The endogenous metabolism of this organism continues in the presence of exogenous glucose. To study the energetics of starvation the ATP contents of Z. anaerobia and P. prévotii have been measured by the firefly luciferase technique. In both cases the ATP content falls rapidly within the initial few hours and then levels out at a small value; it is clearly not related directly to the viability. The capacity of P. prévotii to synthesize ATP during starvation was therefore tested at intervals by adding pulses of suitable energy-yielding substrates such as serine, threonine and pyruvate. The ability to produce ATP on addition of serine is virtually abolished after 42 hours of starvation.

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INTRODUCTION

The initial stages of an investigation of the endogenous metabolism of non-spore-forming anaerobic bacteria have been previously reported by Dawes & Large (1). The principal organisms studied were Zymomonas anaerobia and Peptococcus prévotii as examples of bacteria which obtain their energy by the fermentation of glucose and of amino acids respectively.

It was shown that neither of these organisms possesses reserves of carbohydrate or poly-β-hydroxybutyrate and that the cellular constituent which undergoes the greatest depletion on starvation is ribonucleic acid (RNA). Ultraviolet-absorbing material appears in the suspending fluid concomitantly with RNA depletion, but both processes are suppressed in the presence of magnesium ions.

There was apparently some loss of protein from Z. anaerobia during starvation and ammonia and amino acids were released into the suspending fluid, although the concentration of these materials in the hot-water-extractable pool remained essentially constant. In contrast, there was no significant loss of protein during the starvation of P. prévotii, the concentration of free amino acids in the internal pool was very small, a small amount of ammonia was released by the cells and there was no significant release of amino acids.

The initial analytical studies of cellular composition reported did not include measurements of the ATP content of the cells. As these investigations did not reveal any direct correlation between cellular composition and viability, the work has been continued by studying this aspect of the problem, since it seemed reasonable to assume that continued viability under conditions of starvation must be related in some way, probably in a direct manner, to the availability of energy in the form of ATP.

METHODS

Maintenance and Growth of Organisms

Culture methods. Zymomonas anaerobia (NCIB No. 8227), Zymomonas mobilis (NCIB No. 8938) and Peptococcus prévotii (ATCC No. 14952) were maintained and cultured in the manner previously reported [Dawes & Large (1)].

Viability determinations. These were carried out by the slide culture technique of Postgate, Crumpton & Hunter (2) with the modifications described by us for anaerobic bacteria (1). Further modifications were introduced to obviate contamination which sometimes occurred when using the dry box technique. The slide cultures were prepared inside a small wooden box with a glass top and openings on two opposite sides to permit manipulation. A steady stream of sterile nitrogen was passed through the box throughout the operations and the whole procedure was carried out in a small aseptic cubicle.

Measurement of bacterial density. The method previously described (1) was used.

Preparation of washed suspensions for starvation experiments. After growth the cells were harvested in screw-cap centrifuge bottles previously sterilized by rinsing with acetone and drying under a u.v. lamp. The cell pellets were broken up with a glass rod and the cells suspended in sterile phosphate buffer, pH 6.8. The cell suspensions were bulked together and the cells washed twice with buffer. The resulting cell pellet was ground to a smooth paste with a glass rod and the cells suspended in a suitable volume of buffer. The harvesting procedure was carried out in an aseptic cubicle.

Although early starvation experiments were carried out by distributing suitable volumes of suspension into sterile conical flasks, a modified method was later introduced which used an apparatus described by Dawes & Holms (3). This reduced the time the suspension was in contact with the atmosphere and yielded results which were more consistent than had previously been obtained. The apparatus consisted of a round-bottomed flask fitted with a sintered glass aerator, a tap at the base through which samples were drawn off and a sidearm through which substrates were introduced. After preparation, the cell suspension was immediately transferred to the starvation apparatus and sterile oxygen-free nitrogen bubbled through vigorously to render the atmosphere in the flask anaerobic. After 3 min. the gas flow rate was reduced until nitrogen issued

slowly from the aerator. The flask was then shaken slowly and samples taken when required.

Washing of cells by passage through silicone. Some exploratory experiments were carried out to see whether P. prévotii could be harvested by centrifuging through silicones, as described for Escherichia coli by Hurwitz, Braun & Peabody (4). This technique would expose the cells to much milder treatment than the washing procedures adopted.

Silicones reputed to be equivalent to those reported by the American workers were obtained (Midland Silicones Ltd., MS 2522 and MS 200/5) but experiments showed that neither was suitable alone or mixed, since both had densities less than that of the medium. A denser silicone was obtained (MS 710, density 1.1) which proved satisfactory, yielding compact cell pellets free of contaminating medium.

Cells were harvested in sterile 380 ml. centrifuge bottles and the cell pellets obtained combined in a small volume of used medium. Samples (4 ml.) of the concentrated cell suspension were layered over silicone fluid (3 ml.) in 10 ml. centrifuge tubes, and centrifuged at 19,000 g for 15 min. at 5°C. The supernatant medium was removed by pipette and the silicone layer decanted leaving the cell pellet in the tube.

Preparation of cell-free extracts for disc electrophoresis. Cells of Z. anaerobia (about 70 mg. dry wt.) were suspended in 2.5 ml. ice-cold 67 mM-phosphate buffer, pH 6.8 and disrupted by 1½ min. treatment in an MSE 60 w. ultrasonic disintegrator. The resultant material was centrifuged for 10 min. at 25,000 g to remove whole cells and debris. The supernatant was used for disc electrophoresis. Samples containing 100 µg. of soluble protein were used for each (see Fig. 4).

Polyacrylamide gel electrophoresis. This was performed on a Shandon Disc Electrophoresis apparatus using the techniques described by Davis (5).

Determination of Adenosine Triphosphate (ATP)

The ATP content of the cells was measured by the firefly-luciferase technique, based on the method of Forrest & Walker (6). The apparatus used consists of a high gain photomultiplier tube (E.M.I. type 6097S) the current output of which, after conversion to a voltage, is measured using a Yellow Springs Model 80 laboratory recorder. The high stability voltage supply needed for photomultiplier tube operation is derived from a Nuclear Enterprises type NE 5353 high voltage supply. Using this

apparatus it is possible to measure ATP levels as low as 0.1 µg. in a 1 ml. sample of extract.

Preparation of cell extract for ATP estimations. The cells were centrifuged and the supernatant decanted. The superfluous fluid on the walls of the tube was removed with filter paper and the ATP extracted with 0.3 M-H_2SO_4 (0.25 ml.) for 30 min. at room temperature (about 18°C). The extract was then neutralized with 0.02-M-Na_3PO_4 (4.75 ml.) giving a pH of 7.05. The solution was clarified by centrifuging and the extract stored frozen. Assay of the extracts (1 ml. samples) was carried out within 36 hr. of preparation since significant losses occurred on prolonged storage, and the rate of decomposition on thawing became appreciable.

Preparation of firefly extract. Dried firefly lantern (50 mg. Sigma Chemical Co.) was extracted in a glass manual homogenizer at 0°C with 0.025 M-arsenate-sulphate buffer pH 7.4 (2.5 ml.). The solution was clarified by centrifuging at 60,000 g for 30 min. and kept cold in an ice-bath before and during use. Although it was normal practice to use the extracts immediately after preparation, samples stored frozen for one week showed little loss of activity.

Assay of samples. Cell extract (1 ml.) and $MgSO_4$ (0.375 mg./ml., pH 7.0, 2 ml.) were pipetted into a cuvette and mixed well. Firefly extract (100 µl.) was added quickly from a syringe and the light emission measured 15 sec. after the addition.

Assay of ATP standard. The following solutions were used: (a) H_2SO_4/Na_3PO_4 [0.3 M-H_2SO_4 (0.5 ml.) + 0.02 M-Na_3PO_4 (9.5 ml.), pH 7.05]; (b) $MgSO_4$ (0.75 mg./ml.); (c) 2 mM-phosphate buffer, pH 7.05; (d) ATP solution (1 µg./ml. in 2 mM-phosphate buffer; ATP, disodium salt, from the Sigma Chemical Co. was used). Solutions (a), (b) and (d) (1 ml. of each) were pipetted into a cuvette and mixed well. Firefly extract (100 µl.) was added with a syringe and the light emission measured 15 sec. after the addition.

Chemical Determinations

Glucose. Glucose was determined by the method of Nelson (7).

Pyruvate and 2-oxobutyrate. Pyruvate was determined by the direct method of Friedemann and Haugen (8). 2-Oxobutyrate was also determined by the direct method after an incubation period of 30 min.

Amino acids. These were estimated by the method of Yemm & Cocking (9) as previously described (1).

Manometric methods. Standard Warburg procedures were used.

Cellular carbohydrate. This was estimated by the anthrone method of Trevelyan & Harrison (10) as modified by Binnie, Dawes & Holms (11).

Polyphosphate. This was determined by the method of Harold (12).

Ammonia. This was determined by the modified indophenol method of Chaney & Marbach (13).

RESULTS

Zymomonas mobilis

RNA, viability and cold-acid-soluble nucleotide pool. Although the RNA content of freshly harvested late exponential phase cells of Z. mobilis (average 22% of the dry wt.) is very similar to that of Z. anaerobia (1), marked differences in the rate of degradation of RNA in this organism were observed compared with Z. anaerobia. Fig. 1 shows that after anaerobic starvation of Z. mobilis for 24 hr., the RNA content has fallen from 22% to 10%, and that this degradation is prevented by the presence of 33 mM-$MgCl_2$. Degradation of RNA was accompanied by a release of 260 mμ-absorbing material into the medium, while the cold-acid-soluble nucleotide pool remained essentially constant.

The results of a longer period of starvation, accompanied by study of the effects of RNA degradation on viability, are presented in Fig. 2. In the absence of Mg^{2+}, the period of RNA breakdown falls into two phases: a rapid decline (from 22% to 12%) in the first 24 hr. of starvation, followed by a slower rate of decline (from 12% to 6%) over the next 160 hr. In the presence of Mg^{2+}, there is a much smaller decline (from 22% to 18%) during this period. During the first 70 hr., the presence of Mg^{2+} does not seem to preserve the viability of the cells, but in the period 70-184 hr., the decline in viability does seem to be less in the presence of 33 mM-$MgCl_2$. The 260 mμ-absorbing material seems to be of nucleotide rather than purine nature. This is suggested by the fact that ribose estimations on the supernatant essentially followed the pattern of the 260 mμ-absoring material. The initial burst of nucleotide material released in the first 24 hr. appears to be followed by a slow reutilization in the succeeding 50 hr., since both 260 mμ-absorbing material and ribose disappear from the supernatant during this period. $MgCl_2$ suppresses this release of nucleotides. During the whole of this period the cold-acid-soluble nucleotide pool does not appear to change significantly.

Zymomonas anaerobia

Polyphosphate. This is the only known storage material of bacteria not previously investigated. Neither the cold perchloric acid extract, nor the hot extract contained any non-charcoal-absorbable acid-labile phosphate which would be polyphosphate.

Effect of starvation and magnesium on viability. Z. anaerobia is extremely resistant to anaerobic starvation. Over a period of 145 hr., the viability of the cells fell from about 80% to about 60%. 33 mM-$MgCl_2$ did not appear to exert a significant effect on the viability of Z. anaerobia under these conditions (Fig. 3), although during this time the RNA content fell from 22% to 3%. It is clear that the RNA lost is not essential to the survival of the cell.

Protein content of Z. anaerobia on starvation. A more systematic examination of the effect of starvation on the protein content of Z. anaerobia than that reported previously (1) has been made. Preliminary results had suggested a 10% fall in protein content on 90 hr. starvation (from 70% to 60% of the dry wt.). More extensive examination of the effect of starvation on the protein content of Z. anaerobia suggests that, within the limits of experimental error, there was no significant degradation of protein over a period of 150 hr. starvation.

Attempts have been made to study the qualitative effects of aerobic and anaerobic starvation on individual proteins in Z. anaerobia by examination of the pattern of proteins present in extracts of cells after various periods of starvation by disc electrophoresis on polyacrylamide gel. An example of the type of result obtained is given in Fig. 4.

In extracts of unstarved cells, about 14 protein bands can be distinguished, of which 5 are very prominent. After 159 hr. 13 bands are still evident (Fig. 4) and the same 5 bands are still prominent, although some are less sharply defined. There does not seem to be any major or significant change in individual bands during this time, although quantitative assessment of the amounts of proteins in individual bands is not, as yet, possible. Similar results were obtained when the starvation was carried out anaerobically.

ATP levels during the growth cycle of Z. anaerobia. As a preliminary to study of the effects of starvation on intracellular ATP levels, the levels of ATP in the cells during the growth cycle were investigated. The results are shown in Fig. 5. In general the ATP content increases with the cell density. Whether the changes in slope [similar to those obtained by Forrest & Walker (5)] are significant, is difficult to decide in view of the scatter of the ATP determinations. However, on the basis of several other experiments, it is likely that there is a rapid fall in ATP content as soon as the stationary phase is reached.

ATP levels during starvation. Because of the very rapid fall in ATP content (in the 4 hours immediately after the stationary phase is reached the ATP content falls from 1.3 µg. to 0.1 µg./mg. dry wt.), and because this loss in ATP is accelerated by the processes of harvesting, washing the cells and setting up the flasks for incubation (total time taken for this is usually 2-3 hours), the ATP

levels recorded at the beginning of a starvation period never exceeded 0.13 µg./mg. dry wt. During the succeeding 2-3 hours this level fell to 0.06 µg./mg. dry wt., and then declined further in the succeeding 50 hr. to 0.015 µg./mg. dry wt. This seems to represent a basal level, since it did not decline further in the next 100 hr. starvation.

Peptococcus prévotii

Ammonia and amino acid pools during starvation. Washed suspensions, harvested after 24 hr. growth, were starved at 37° under nitrogen for 94 hr. At intervals determinations of viability by the slide culture method, protein content, amino acid pool and ammonia content of both pools and supernatants were made. The amino acid content of the pools was negligible; values for the other parameters are shown in Fig. 6.

After an initial slight fall, which may not be of significance, the protein content of the cells remained constant throughout the period of starvation, while the percentage viability fell from 91 to 20. Ammonia was released into the suspending fluid, reaching a plateau value by about 30 hr., and the ammonia of the pool went through a maximum at 22 hr. It seems probable that most of this ammonia arises from deamination of bases. We have already reported on the stability of the bacterial protein during prolonged starvation.

Metabolism of glucose by P. prévotii. We have previously reported that cells of P. prévotii grown for 42 hr. did not metabolize glucose. However, it was subsequently discovered that cells harvested after 24 hr., and still in their active phase of growth, did so to a limited extent under anaerobic conditions. Washed suspensions were therefore incubated with [U-^{14}C]glucose in the Warburg apparatus under nitrogen and the gas exchange, glucose utilization and fate of the radioactivity were studied. Typical results are given in Table 1. Approximately one third of the added glucose was utilized as measured by the Nelson method and the disappearance of radioactivity from the assayed supernatant. Of this, 8.3% appeared in the respiratory CO_2. An endogenous release of CO_2 was noted, and when correction was made for it the CO_2 released represented 8% of the added glucose carbon, a figure which agrees well with the value obtained radiochemically. If correction is not made for endogenous CO_2 release, then the percentage conversion of glucose carbon to CO_2 is calculated as 14.5%, a value widely divergent from the radiochemical estimate. We conclude therefore that endogenous metabolism continues in the presence of exogenous glucose.

The discrepancy between the loss of radioactivity from the supernatant and that appearing in the CO_2 is possibly due to loss of volatile products during the plating procedure. The cells were not assayed, however, and it is possible that some assimilation had occurred.

Under aerobic conditions no significant utilization of glucose occurred.

ATP content of P. prévotii. It was a general observation that cells harvested in the stationary phase had a lower ATP content than those from the phase of active growth; the longer cells were left in the stationary phase the lower became the ATP content. Cells were therefore harvested at 24 hr., during growth, for experiments on the ATP content. It was observed that the harvesting and washing procedures also result in a decrease in the ATP content of the cells.

Effect of starvation on the ATP content and viability. Over a period of 44 hr. it was found that the ATP content declined to a plateau value and viability also declined, although much less rapidly (Table 2). Experiments were then carried out to study the ATP content during the initial stages of starvation, i.e. during the period when rapid changes occurred. Fig. 7 reveals that a rapid decrease in ATP content occurs during the first hour of starvation followed by a much slower decline. In these experiments it was clear that the initial ATP content showed variation with each batch of cells, although the general pattern of behaviour was common to all.

Effect of agitation with air and nitrogen on the ATP content. Cell suspensions were starved for short periods while the cells were agitated by bubbling air or nitrogen through them. It will be seen from Fig. 8 that aeration produced a fall in ATP content which quickly attained a plateau value. Agitation with nitrogen, however, produced an initial rapid increase in ATP content followed by a decline. This pattern of behaviour was observed in a number of experiments and it is concluded that agitation of the cells with nitrogen in some way permits an immediate increase in their ATP content.

Effect of various metabolites on the ATP content. Preliminary experiments had shown that the absolute ATP content of the cells could not be correlated directly with the viability of the cells and it seemed much more likely that survival might be linked with the capacity of cells for synthesis of ATP from a suitable source of endogenous energy. To test the capacity of starved cells for ATP synthesis it was decided to examine their response to the addition of a number of different compounds. Amino acids and glucose were initially selected for this purpose.

The ability of our strain of P. prévotii to metabolize serine, threonine, histidine, glutamine and glutamic acid was first tested manometrically by measuring total gas evolution. There was no appreciable gas evolution from histidine, glutamate or glutamine, but extremely rapid and vigorous fermentation of serine and threonine occurred. Pyruvate, as a product of serine deamination, was also investigated. Hydrogen evolution is shown in Fig. 9 for serine, threonine and pyruvate. It will be observed that pyruvate was also rapidly fermented.

(a) Glucose. As previously noted, cells grown for 24 hr. metabolize glucose to a limited extent whereas those grown for 42 hr. do not. The effect of a pulse of 5 mM-glucose after $2\frac{1}{2}$ hr. of starvation is shown in Fig. 10. There is an immediate, rapid increase in the ATP content of the cells and this behaviour was confirmed in several experiments. Cells which, by manometric evidence, were judged incapable of metabolizing glucose showed no increase in ATP content when treated with a glucose pulse.

The effect of a second pulse of glucose on responsive cells was also tested (Fig. 11). This produced a second rapid increase in ATP content although obviously very little glucose was being metabolized and this apparently only in the initial stages of the first addition. It should be noted however, that the total increase in ATP observed corresponds to only 0.24 mμmole ATP per ml. of suspension which, if one assumed that Embden-Meyerhof glycolysis occurred, would correspond to only 0.12 mμmole of glucose fermented per ml., an amount not detectable by the Nelson method of estimation.

(b) Serine. Fig. 12 shows that a pulse of 5 mM-L-serine results in a very rapid increase in ATP content which, however, also decreases very rapidly. If however the added L-serine concentration is increased to 20 mM the ATP content reaches a higher level and the rapid decrease is not observed (Fig. 13). Ammonia is produced and the pyruvate concentration in the supernatant decreases. The addition of more L-serine gives a further increase in ATP content and the pyruvate concentration of the supernatant increases.

(c) Threonine. The effect of a pulse of 5 mM-DL-threonine on starving cells is shown in Fig. 14. Estimations of amino acid and ammonia in the supernatant were also carried out. Again the pattern of rapid increase and decrease was observed.

(d) Pyruvate. Deamination of serine yields pyruvate and it is presumably the further metabolism of pyruvate which yields energy to the cell. The effect of a pyruvate pulse (5 mM) is seen in Fig. 15 and the maximum ATP content coincides with the virtually complete utilization of pyruvate.

(e) 2-Oxobutyrate. This compound is the product of threonine deamination and addition of it to a starving culture results in a rapid increase in ATP content (Fig. 16).

(f) Glutamate. Manometric experiments indicated that glutamate was not fermented and it was found that addition of L-glutamate to a starving suspension resulted in either no change or a decrease in ATP content.

Capacity of cells for ATP production during prolonged starvation. From the foregoing survey with various substrates it is evident that, in the early stages of starvation, cells respond rapidly to the addition of an energy-yielding substrate as judged by a marked increase of ATP content. The effect of prolonged starvation on the capacity to respond to pulses of L-serine was therefore investigated. Cells were starved for 42 hr. and at intervals samples were withdrawn from the suspension and tested with 10 mM-L-serine. Fig. 17 reveals that both the rate and extent of the response declined as starvation progressed and that by 42 hr. the response was barely detectable. Simultaneous viability determinations were not carried out in these preliminary experiments and work is now continuing to establish whether a positive correlation is possible between survival and the ability to synthesize ATP on the addition of an exogenous substrate.

Experiments over a period of 24 hr. have revealed that some batches of cells do not show a marked decrease in the extent of ATP production during this time although the rate at which ATP is synthesized decreased in the sequence: 5 hr., 0.017 µg./min.; 10 hr., 0.015 µg./min.; 20 hr., 0.0069 µg./min.; 24 hr., 0.00375 µg./min.

DISCUSSION

The two organisms of the genus Zymomonas we have investigated, Z. mobilis and Z. anaerobia, display similar metabolic pathways for glucose, and other similarities. Although their RNA contents during exponential growth are similar, their behaviour on starvation is quite different. The rate of RNA loss in Z. anaerobia is relatively slow, e.g. a decline from 22% of the dry weight to 12% takes 90 hr., (Fig. 3) while in Z. mobilis (Fig. 2) this change takes place in 24 hr. Moreover, while the loss of RNA in Z. anaerobia is essentially linear, that in Z. mobilis displays a rapid initial fall followed by a much slower rate of decline. The percentage viability of Z. mobilis does not seem to differ significantly from that of Z. anaerobia during the initial 90 hr., and $MgCl_2$ apparently does not have any protective properties, although it does suppress RNA degradation. Both organisms are rather resistant to prolonged starvation, and this resistance does not appear to be related to their high RNA content.

The stability of overall protein content of Z. anaerobia does not seem to mask any kind of turnover or preferential degradation of one protein and resynthesis of another, since the pattern of individual proteins in crude supernatant fractions remains the same even after 160 hr. starvation.

In P. prévotii, the stability of protein during starvation has been further confirmed and the ammonia found in the intracellular pool and released by the cell most probably arises by the deamination of nucleotide bases. In all experiments carried out the free amino acid pool of the organism has been extremely small and, in view of the stability of cellular protein, our earliest conjecture that free amino acids might prove to be the substrates for endogenous metabolism now seems untenable.

The ability of cells harvested after 24 hr. growth to metabolize glucose was exploited to investigate the status of the endogenous metabolism of P. prévotii in the presence of an exogenous substrate. Combined manometric and isotopic experiments with [U-^{14}C]glucose revealed that endogenous metabolism continues in the presence of added glucose. As yet, we know nothing of the pathway of glucose metabolism which occurs in this organism.

Our inability to correlate changes in the cellular content of protein, carbohydrate or RNA with the viability of these organisms led us to consider the ATP content of the cells as an index of the availability of energy. Application of the very sensitive firefly

luciferase system to bacterial extracts has enabled us to measure extremely small amounts of ATP in the cells. The ATP content of Z. anaerobia was found to increase during exponential growth and to fall extremely rapidly on starvation. Thus within 4 hr. the ATP content was but 4.5% of the value observed at the end of the exponential growth phase and it had fallen to 1% in 60 hr. It then remained constant at a very low value (0.014 µg./mg. dry wt.) for a further 100 hr. of starvation, and over the total period of 160 hr. the viability fell from 80% to 55%.

Cells of P. prévotii also had a higher ATP content during exponential growth than in the stationary phase and the harvesting and washing procedures led to a further decrease. Experiments with starving cells revealed that the ATP content fell rapidly in the initial stages (1-2 hr.) and then declined much more slowly. Although the viability also declined, this was at a much slower rate than the fall in ATP content.

A curious, though reproducible, feature was observed when starving suspensions were agitated with nitrogen; the ATP content increased rapidly and then declined. The explanation is obscure but possibly agitation facilitated the diffusion from the cells of compound(s) which endogenously suppressed ATP formation. Agitation with oxygen, however, resulted in a fall in ATP content.

It appeared to us that the ATP content per se was probably not directly correlated with viability, but a much more likely correlation might be found between viability and the capacity of the starving cell to produce ATP from endogenous energy supplies. Since, in the absence of precise knowledge of the endogenous energy-yielding processes, we are unable to control or furnish cellular energy directly, recourse must be had to supplying exogenous energy sources and measuring the response of the ATP-synthesizing systems. This method clearly has its limitations, for permeability might well become a limiting factor, especially after prolonged starvation. However, we have investigated this method of assessment as a first approach to the problem.

It was observed manometrically that serine, threonine and pyruvate were all fermented rapidly by washed suspensions. The addition of pulses of these compounds (and also 2-oxobutyrate, the product of threonine deamination, and glucose) to suspensions starved for short periods resulted in immediate, rapid increases in the ATP content of the cells. The ATP reaches a maximum and then usually displays a rapid decline, the rate of which suggests that either an ATPase or some transferase system is active. With serine and glucose the effects of second pulses of substrate and of increased substrate concentrations were studied. It was found that the concentration of

ATP could be increased by these devices and that, in the presence of a high serine concentration (20 mM), the ATP content reached a plateau value and did not display the rapid decrease observed with pulses of 5 mM-serine.

Since the initial step in the metabolism of serine and threonine is deamination, yielding pyruvate and 2-oxobutyrate respectively, and deamination is not an energy-yielding process, the effects of pyruvate and 2-oxobutyrate on the ATP level of the cells were studied. Although keto acids frequently present permeability problems there was no evidence of this with P. prévotii at pH 6.8 and both substrates resulted in a rapid increase in ATP content.

Having established the response of the cells starved for short periods to such pulses of energy-yielding substrates, the investigation was then extended to the effect of much longer periods of starvation. Serine was used as the test substrate and it was found that, as starvation was prolonged, both the rate and the extent of the response, as measured by ATP production, decreased until, usually by 42 hr., the effect of serine was barely detectable. Further experiments are being undertaken to study the viability of the cells over these experimental periods.

The insignificant ATP production when cells are treated with serine after 42 hr. starvation may not, of course, reflect the true state of the energy-yielding and utilizing systems of the cell. It may simply reflect a changed permeability to an exogenous substrate as a result of starvation, or a changed response specific to serine. It is hoped that similar experiments with different substrates may help us to decide between these possibilities.

It is appreciated that measurements of ATP content alone cannot be so informative as determinations of the ATP/ADP ratios of cells at any given phase of starvation. Methods are therefore being worked out to enable this additional information to be obtained.

LITERATURE CITED

1. Dawes, E. A. & Large, P. J. (1965). The Endogenous Metabolism of Anaerobic Bacteria. Final Technical Report, December 1965 for Contract No. DA-91-591-EUC-3608. (European Research Office, U.S. Army).

2. Postgate, J. R., Crumpton, J. E. & Hunter, J. R. (1961). J. gen. Microbiol. 26, 15.

3. Dawes, E. A. & Holms, W. H. (1958). Nature (Lond.) 178, 318.

4. Hurwitz, C., Braun, C. B. & Peabody, R. A. (1965). J. Bact. 90, 1692.

5. Davis, B. J. (1964). Ann. N.Y. Acad. Sci. 121, 404.

6. Forrest, W. W. & Walker, D. J. (1965). J. Bact. 89, 1448.

7. Nelson, N. (1944). J. biol. Chem. 153, 375.

8. Friedemann, T. E. & Haugen, G. E. (1943). J. biol. Chem. 147, 415.

9. Yemm, E. M. & Cocking, E. C. (1955). Analyst 80, 209.

10. Trevelyan, W. D. & Harrison, J. S. (1952). Biochem. J. 50, 298.

11. Binnie, B., Dawes, E. A. & Holms, W. H. (1960). Biochim. biophys. Acta 40, 237.

12. Harold, F. M. (1960). Biochim. biophys. Acta 45, 172.

13. Chaney, A. L. & Marbach, E. P. (1962). Clin. Chem. 8, 130.

Table 1. Anaerobic utilization of [U-^{14}C]glucose by washed suspensions of P. prévotii

Cells were harvested after 24 hr. growth and made into a suspension (4.8 mg./ml.) in phosphate buffer, pH 6.8.
Warburg vessels contained: cell suspension, 2.5 ml.; [U-^{14}C]glucose (5.16 µmoles, 5962 x 10^3 counts/Ksec.), 0.3 ml. in side arm; 10% (w/v) NaOH, 0.2 ml. in centre well. Water replaced glucose in the endogenous controls. Atmosphere, nitrogen; temperature 37°. Double side arm vessels were used for CO_2 evolution measurements, with 0.3 ml. of 5N-H_2SO_4 in the second side arm, and NaOH omitted from the centre well.
At zero time and after 265 min. vessels were removed, the cells separated by centrifuging, and analyses for glucose and radioactivity performed. The contents of the centre wells were diluted to 20 ml. with CO_2-free water and portions (0.1 and 0.2 ml.) plated for radioactivity determinations.

Time (min.)	Glucose concn. mM	Glucose utilized per cent	Radioactivity counts/Ksec. x 10^{-3}	Radioactivity utilized per cent	Total CO_2 released µmoles	Endogenous CO_2 released µmoles	Corrected CO_2 from glucose µmoles*	Radioactivity in CO_2 counts/Ksec. x 10^{-3}	Radioactivity in CO_2 per cent of added activity
0	1.87	-	5962	-	-	-	-	-	-
265	1.28	31.5	4414	35.9	4.52	2.03	2.49	4594	8.3

* Since 5.16 µmoles glucose ≡ 30.96 µatoms C, the percentage of glucose converted to CO_2 is $\frac{2.49}{30.96} \times 100 = 8.04$. If no correction is made for endogenous CO_2 output, i.e. an evolution of 4.52 instead of 2.49 µmoles, then the percentage conversion of glucose to CO_2 is $\frac{4.52}{30.96} \times 100 = 14.6$, which reveals a considerable discrepancy with the percentage of added radioactivity found in the CO_2 (8.3).

Table 2. ATP content of P. prévotii during anaerobic starvation.

Cells were harvested after 24 hr. growth and suspended in 150 ml. of phosphate buffer, pH 6.8, at a density of 1.23 mg./ml. Samples (10 ml.) were withdrawn at intervals for ATP estimations, and at the same time viabilities by slide culture were determined.

Period of starvation (hr.)	ATP content µg./mg. dry weight	Percentage viability
0	0.212	94
18	0.020	52
25	0.014	40
44	0.013	37

Fig. 1. Effect of Mg^{2+} ions on the degradation of RNA by anaerobically starved Z. mobilis.

Cells of Z. mobilis from late exponential phase (10 mg. dry wt.) were harvested aseptically and resuspended in 10 ml. of sterile 67 mM-phosphate buffer, pH 6.8, in 50 ml. conical flasks under N_2 with and without 33 mM-$MgCl_2$. Flasks were removed at intervals, cells centrifuged and extinction of the supernatant at 260 mμ measured. Cells were fractionated into cold 0.7 N-$HClO_4$-soluble nucleotide pool and RNA, and estimated as described previously (1). Results expressed as % of dry wt. of cells using yeast RNA as standard. RNA content: ■, in presence of $MgCl_2$; □, in absence of $MgCl_2$. Cold acid-soluble-nucleotide pool: ●, in presence of $MgCl_2$; ○, in absence of $MgCl_2$. 260 mμ-absorbing material: ▲, in presence of $MgCl_2$; △, in absence of $MgCl_2$.

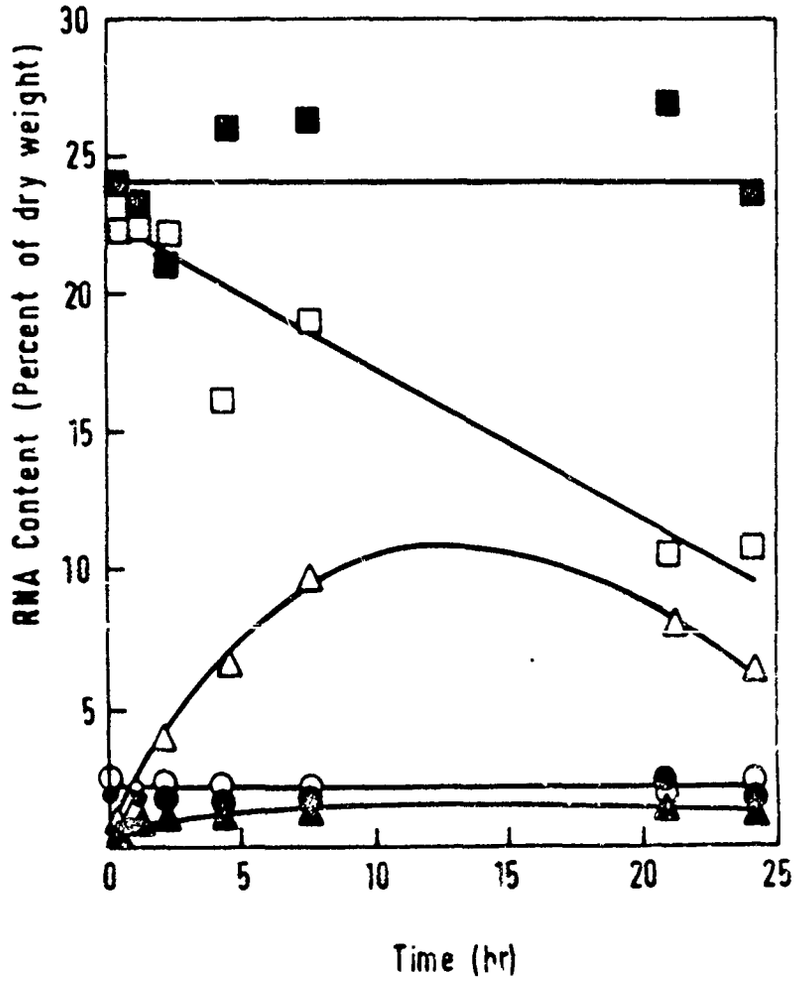


Fig. 2. Effect of Mg^{2+} ions on viability and RNA content of Z. mobilis starved anaerobically.

Conditions were as for Fig. 1, except that starvation was carried on for longer times and samples were also removed for viability determination by the slide culture technique. RNA content: ■ in presence of; □, in absence of $MgCl_2$. 260 mμ-absorbing material: ▲ in presence of; △, in absence of $MgCl_2$; ribose: ▽, in absence of $MgCl_2$. Viability: ◐, in presence of; ○, in absence of $MgCl_2$.

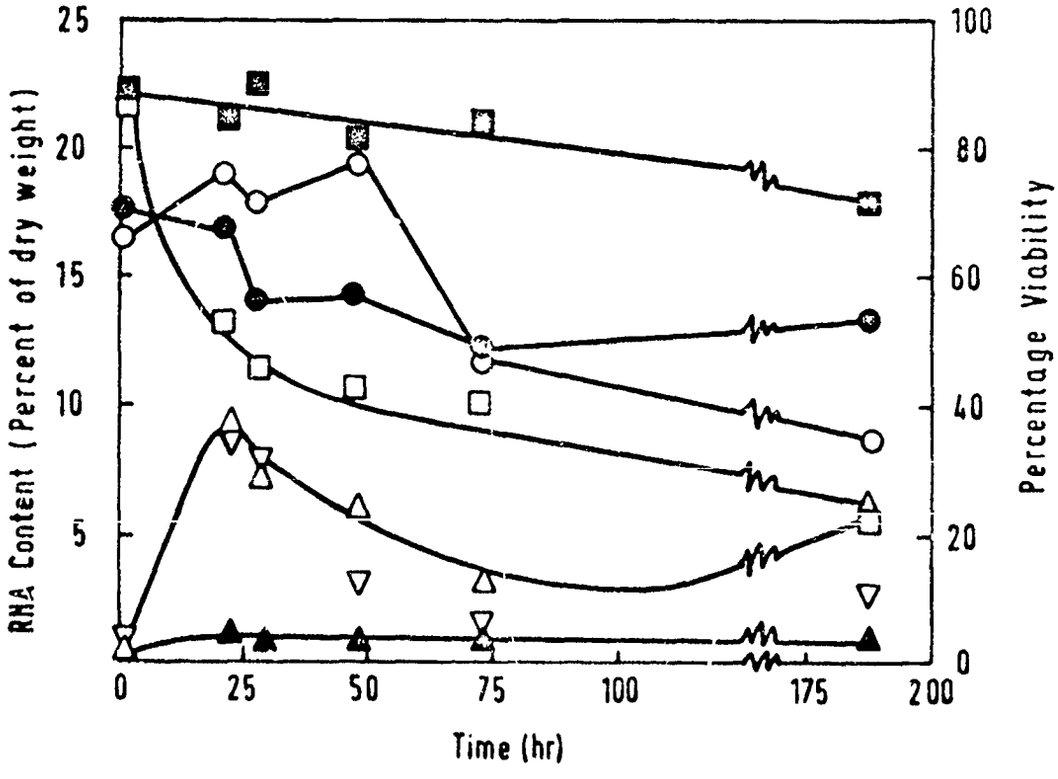


Fig. 3. Effect of Mg^{2+} ions on viability and RNA content of Z. anaerobia starved anaerobically.

Conditions were as for Fig. 2. RNA content: ■ , in presence of; □ , in absence of $MgCl_2$. Viability: ● , in presence of; ○ , in absence of $MgCl_2$.

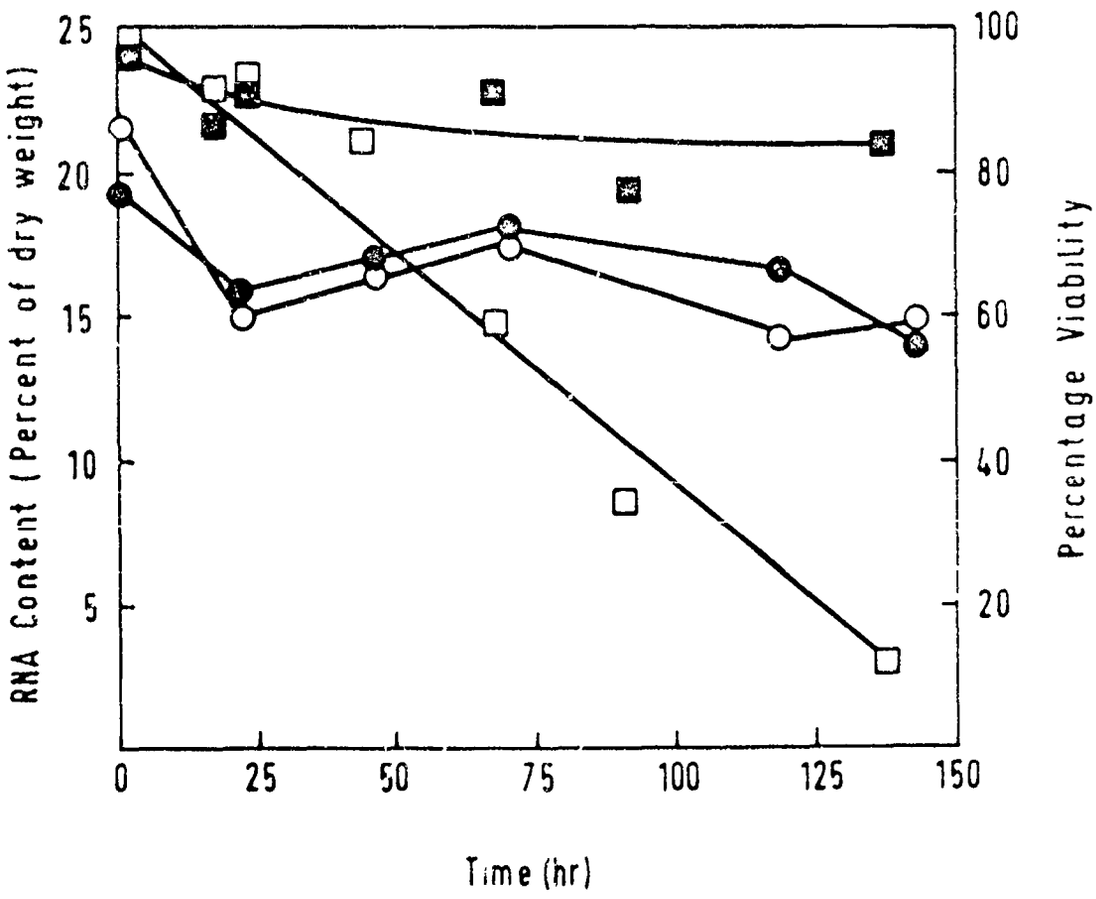
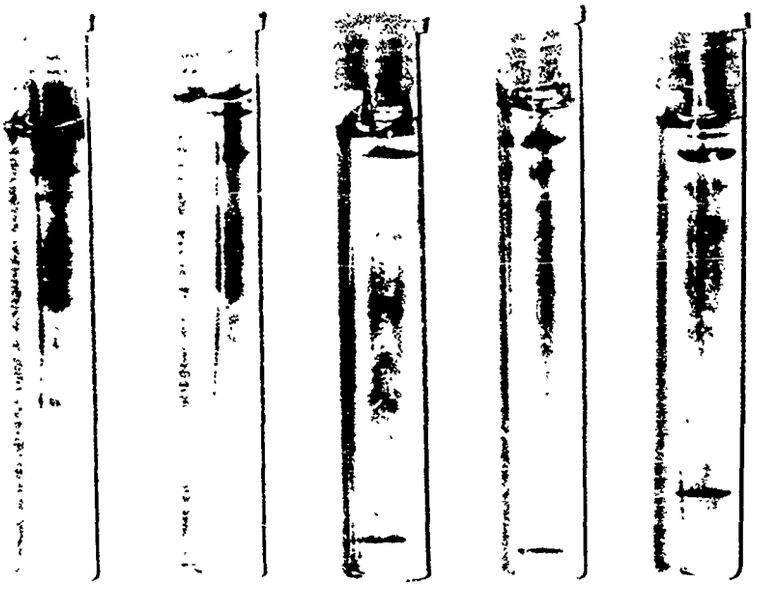


Fig. 4. Effect of starvation on the soluble protein of Z. anaerobia.



Starvation Time (hr.)	0	21	44	111	159

Cell-free extracts of Z. anaerobia were prepared as described in the text after starvation in air for various lengths of time as indicated. Samples of the extracts containing 100 µg. soluble protein were submitted to electrophoresis at pH 9.5 on polyacrylamide gel (5), and stained with 1% (w/v) amido black in 7% v/v acetic acid.

Fig. 5. Growth and ATP content of Z. anaerobia.

●, Growth, measured spectrophotometrically at 570 mμ;
▲, ATP content, measured as described in the text.

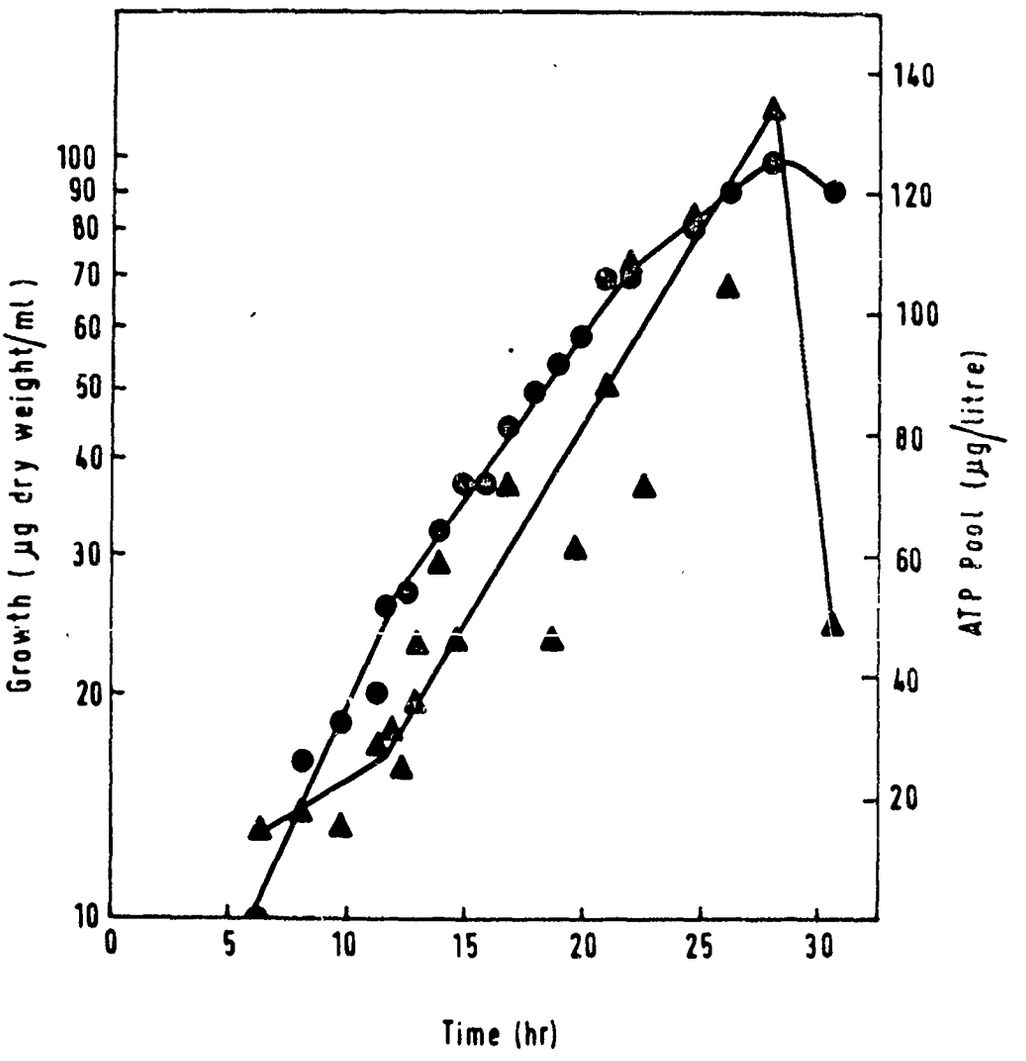


Fig. 6. Protein content, viability and ammonia release during prolonged starvation of Peptococcus prévotii.

Cells were harvested aseptically after 24 hours growth, washed with sterile 67 mM-KH_2PO_4 buffer, pH 6.8, and resuspended in 150 ml. of the same buffer at a density of 1.17 mg./ml.. The suspension was placed in a starvation apparatus and made anaerobic by gassing with sterile oxygen-free nitrogen. The suspension was maintained at 37° and □, viability; ■, protein content; ○, ammonia in supernatant; and ●, ammonia content of cells determined at intervals.

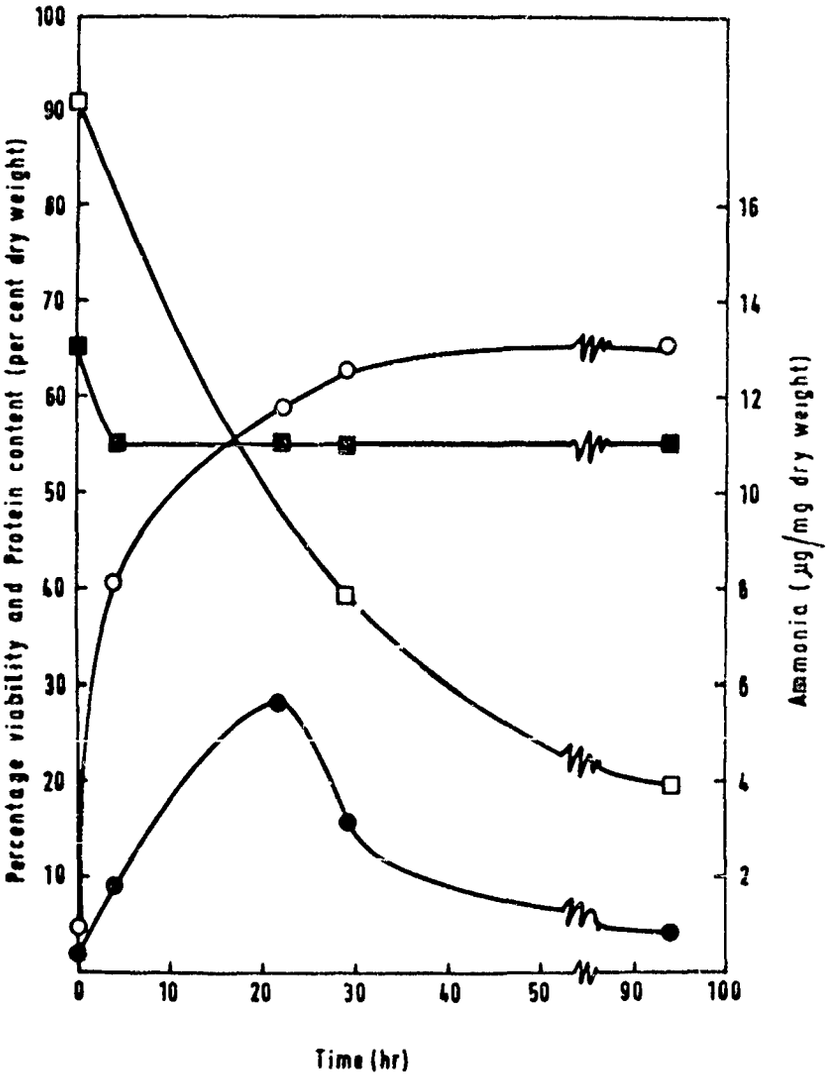


Fig. 7. ATP content of Peptococcus prévotii during starvation.

Cells were harvested with aseptic precautions after 24 hours growth, washed with sterile 67 mM-KH_2PO_4 buffer, pH 6.8, and resuspended in the same buffer at a density of 1.41 mg./ml.. The suspension was placed in a starvation apparatus and made anaerobic by gassing with sterile oxygen-free nitrogen. The suspension was shaken and samples taken for ATP assay at intervals.

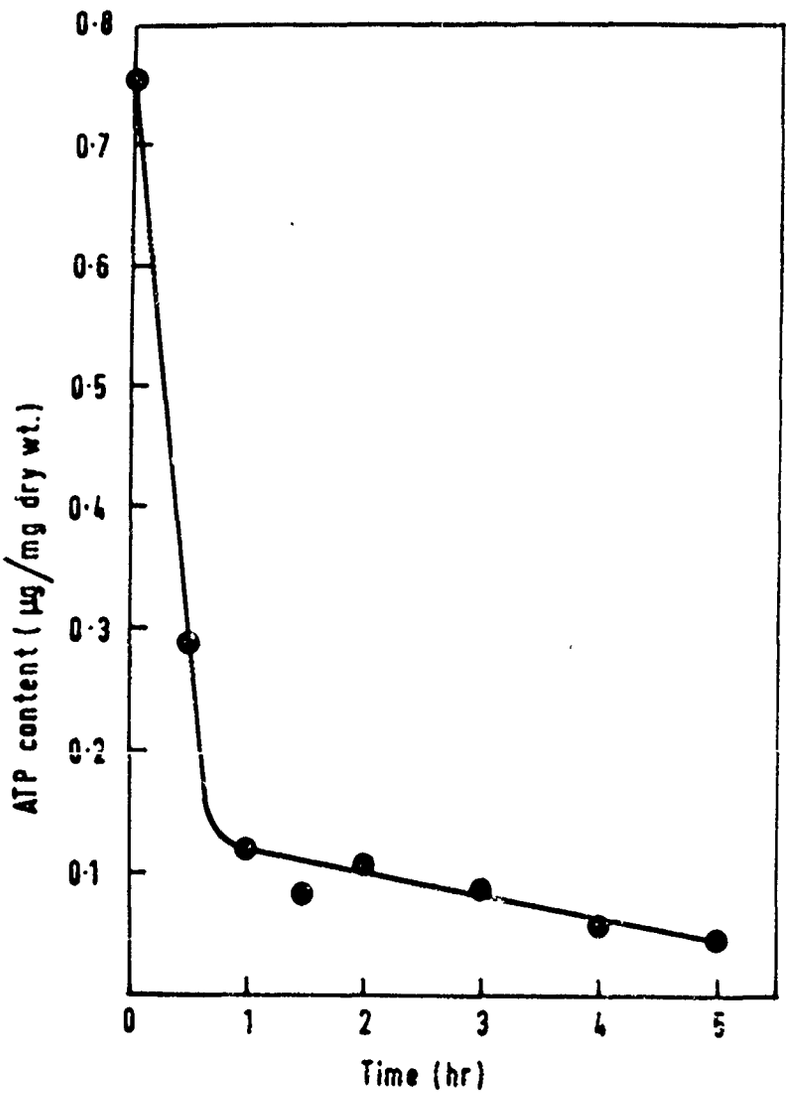


Fig. 8. Effect of agitation with oxygen and nitrogen on the ATP content of Peptococcus prévotii.

Cells were harvested aseptically after 24 hours growth, washed with sterile 67 mM-KH_2PO_4 buffer, pH 6.8, and resuspended in 100 ml. of the same buffer at a density of 1.65 mg./ml.. A sample was taken for ATP assay and the remaining suspension divided into two equal volumes. Air and oxygen-free nitrogen were passed through (1 l./min.) the suspensions which were shaken in 250 ml. Erlenmeyer flasks on a Griffin flask shaker at 250 oscillations/min. and maintained at 37°.

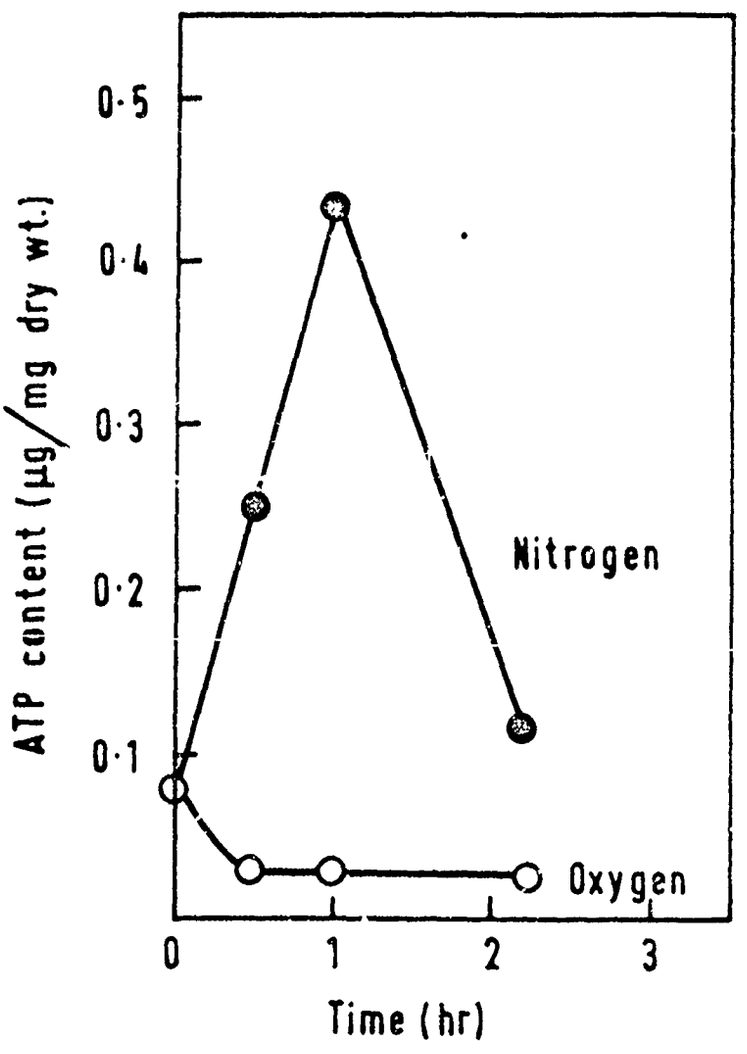


Fig. 9. Hydrogen evolution during the utilization of L-serine, DL-threonine and pyruvate by Peptococcus prévotii.

A washed suspension of the organism was prepared and Warburg flasks set up with the following contents: bacterial suspension (2.8 mg./ml.), 2.5 ml.; substrate (33.3 mM, pH 7.0), 0.3 ml.; and, in centre well, KOH (20% w/v), 0.2 ml.. Gas phase: oxygen-free nitrogen. Temperature 37°. Shaking rate: 120 oscillations/min.. L-serine, □; DL-threonine, ● ; pyruvate, ○ .

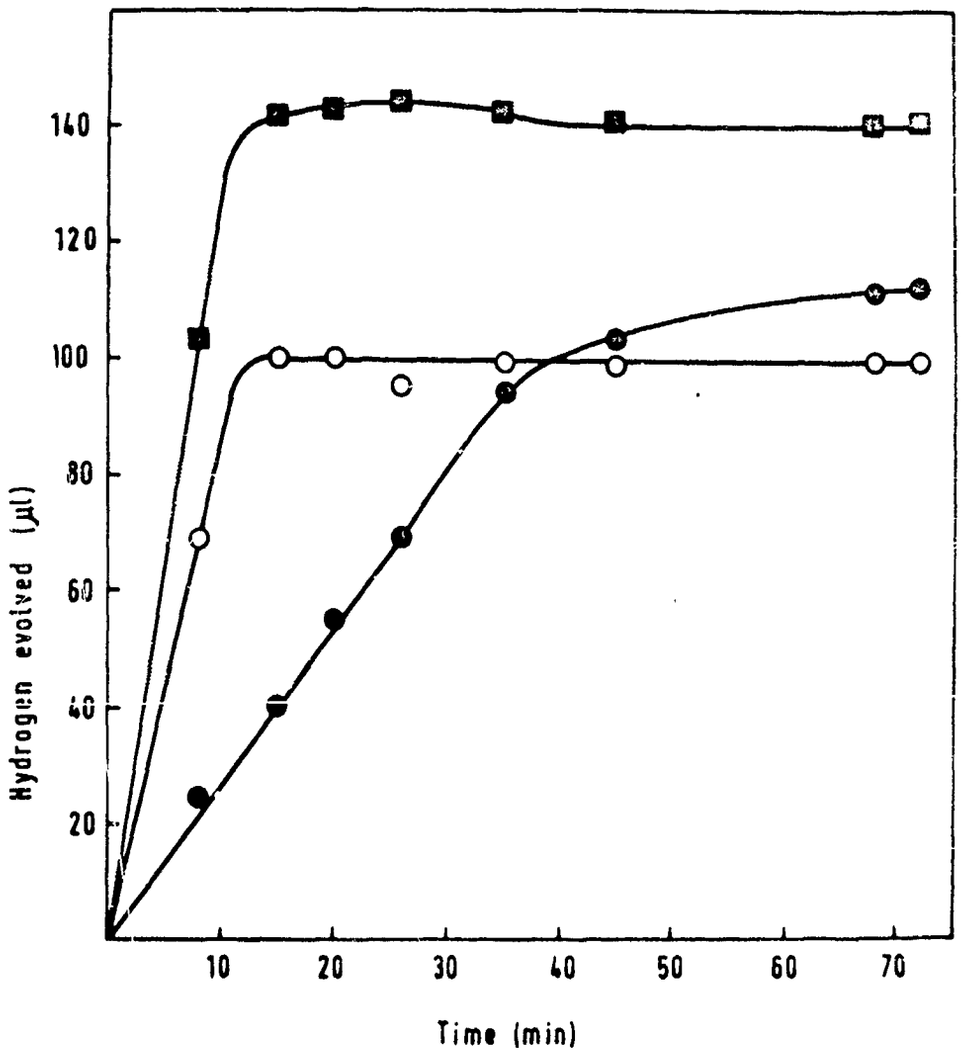


Fig. 10. Effect of glucose on the ATP content of starved Peptococcus prévotii.

Cell density, 3.15 mg./ml.; final substrate concentration, 5 mM. The substrate was added after 2 hr. 35 min. starvation.

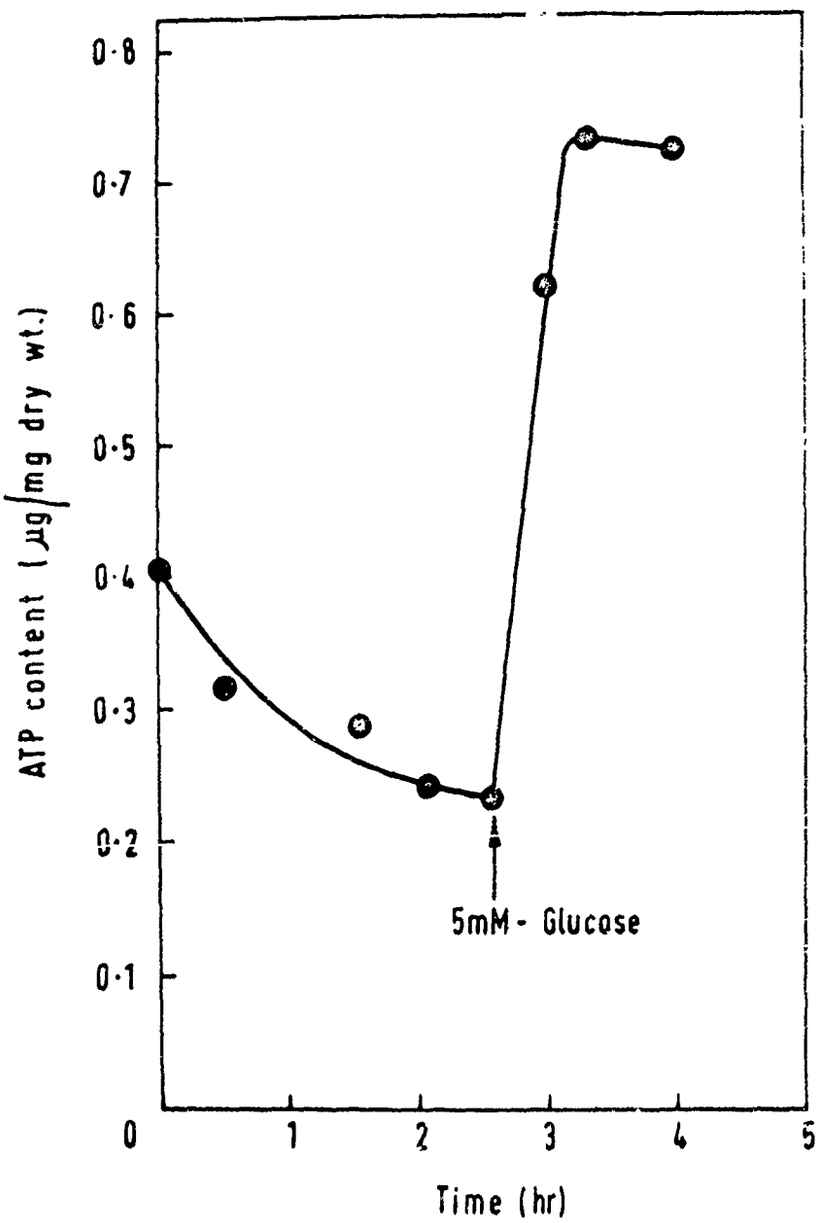


Fig. 11. Effect of a second addition of glucose on the ATP content of starved Peptococcus prévotii.

Cell density, 1.2 mg./ml.. Glucose to a final concentration of 6 mM was added after 3 hr. 15 min. starvation. A second addition was made after 7 hr. 5 min. starvation to give a final glucose concentration of 12.6 mM. ATP content, ●; glucose in supernatant, ■.

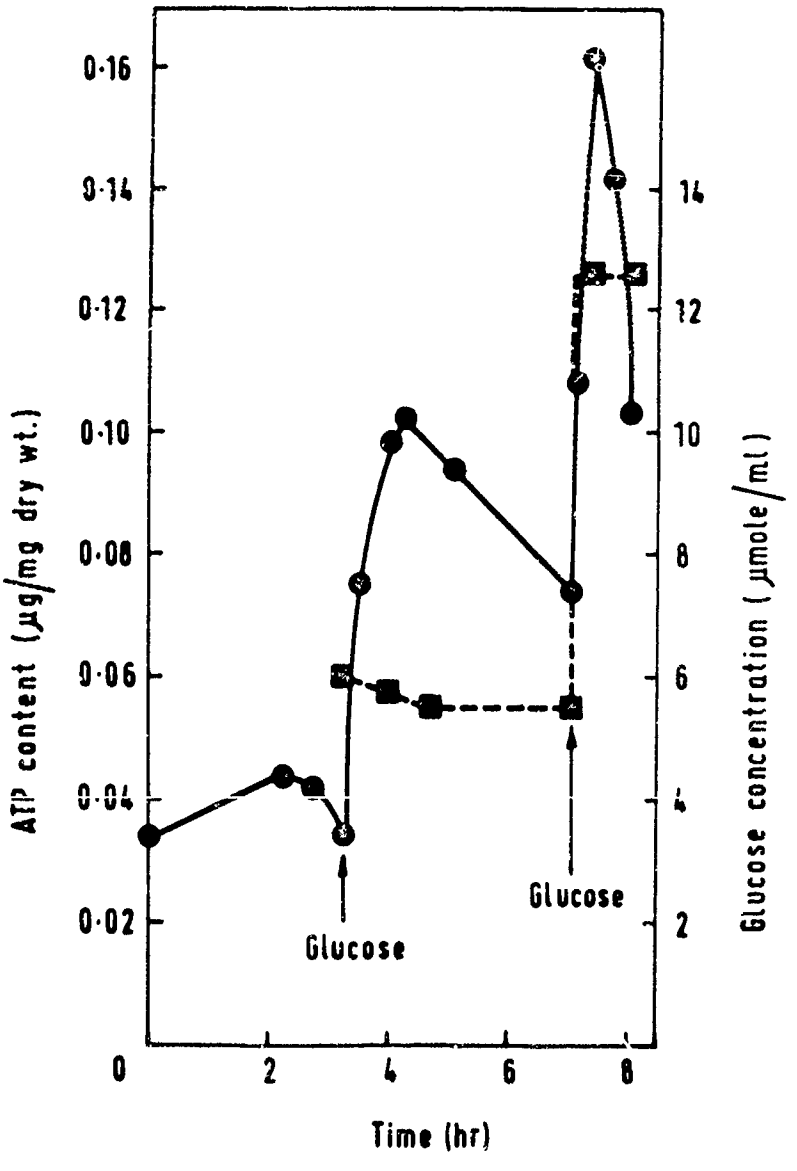


Fig. 12. Effect of L-serine on the ATP content of starved Peptococcus prévotii.

Cell density, 1.05 mg./ml.; final substrate concentration, 5 mM. The substrate was added after 2 hr. 50 min. starvation.

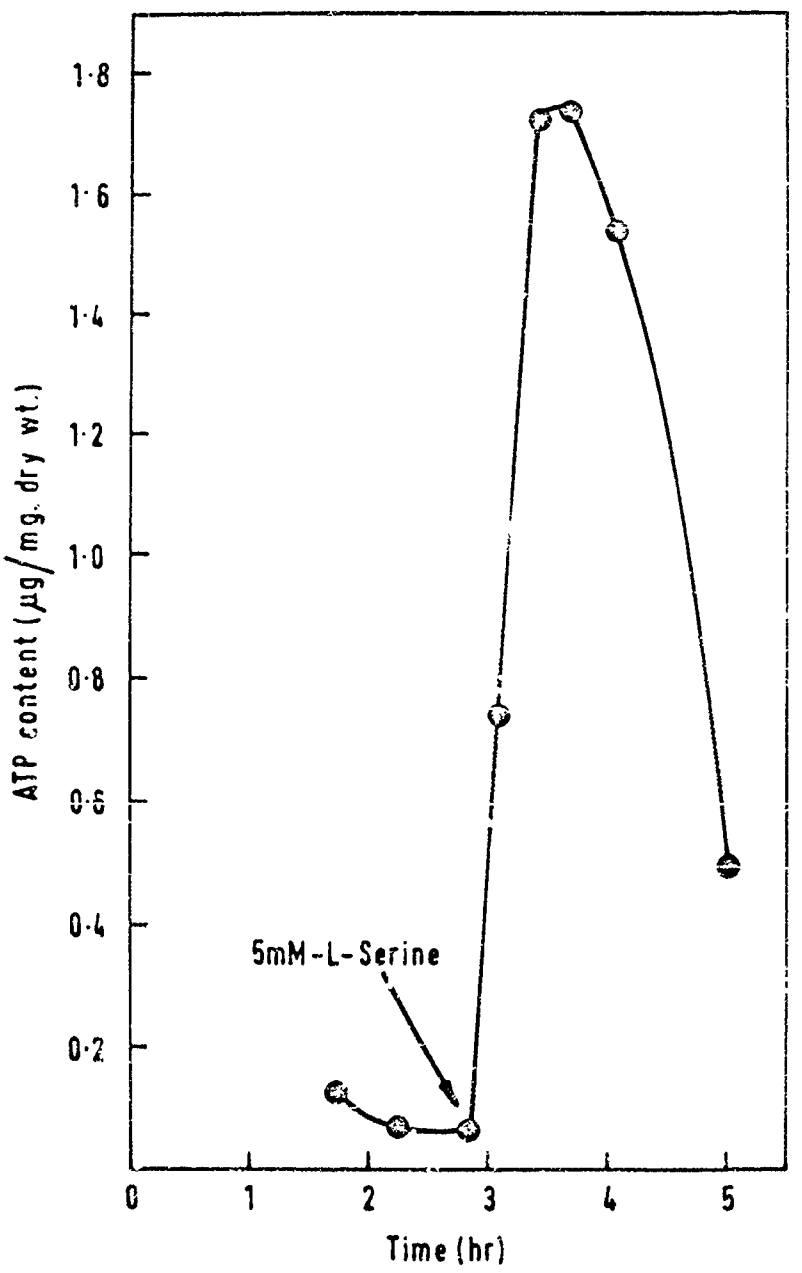


Fig. 13. Effect of a second addition of L-serine on the ATP content of starved Peptococcus prévotii.

Cell density, 0.8 mg./ml.. Serine was added after 2 hr. 15 min. to give a final concentration of 20 mM. A second serine addition was made after 3 hr. 35 min. to give an increase of 10 mM. ATP content, ●; ammonia formed, ■; pyruvate formed, ○.

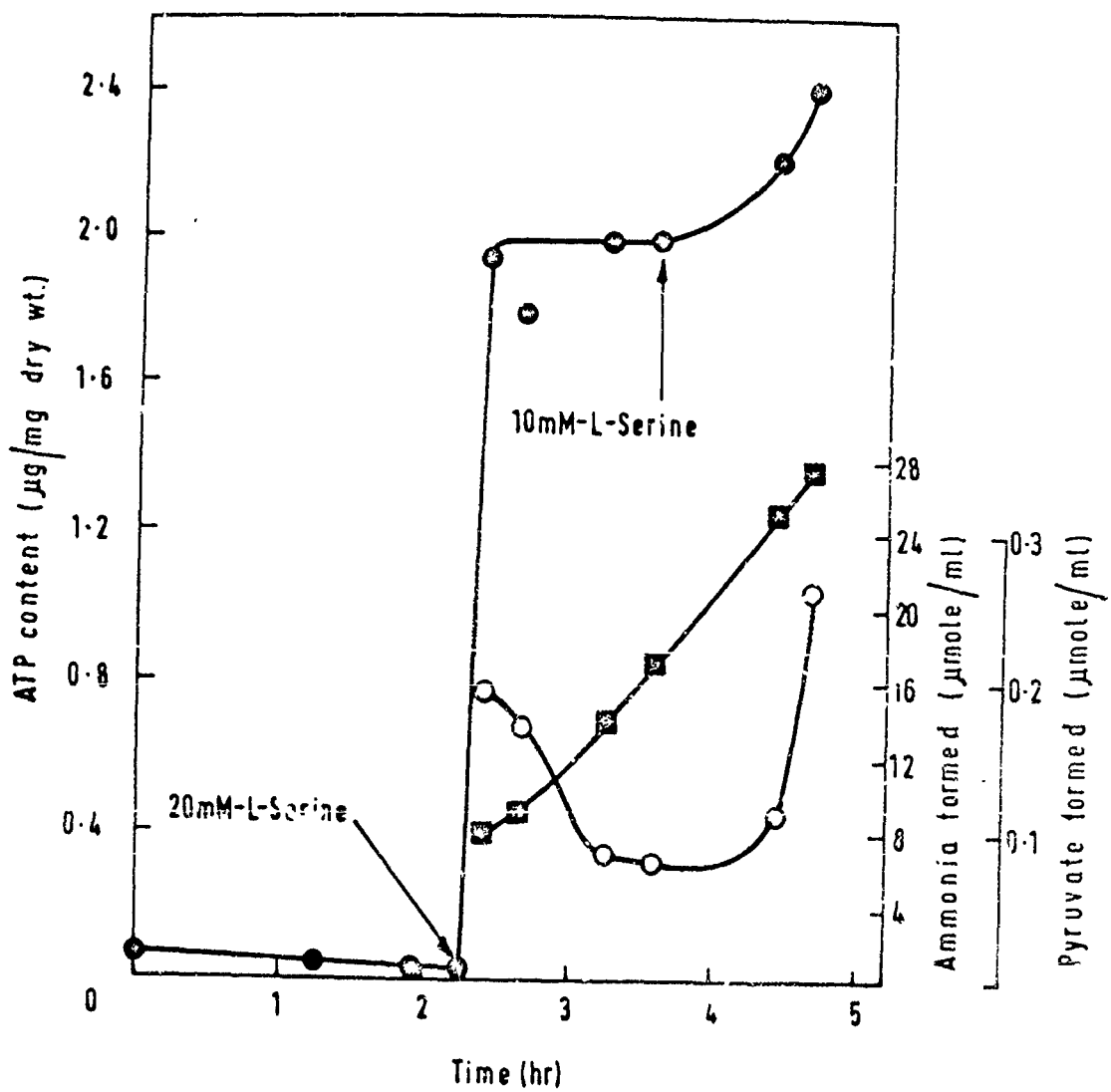


Fig. 14. Effect of DL-threonine on the ATP content of starved *Peptococcus prévctii*.

Cell density, 1.65 mg./ml.. Threonine to a final concentration of 5 mM was added after 2 hr. 30 min. starvation. ATP content, ●; ammonia formed, ■.

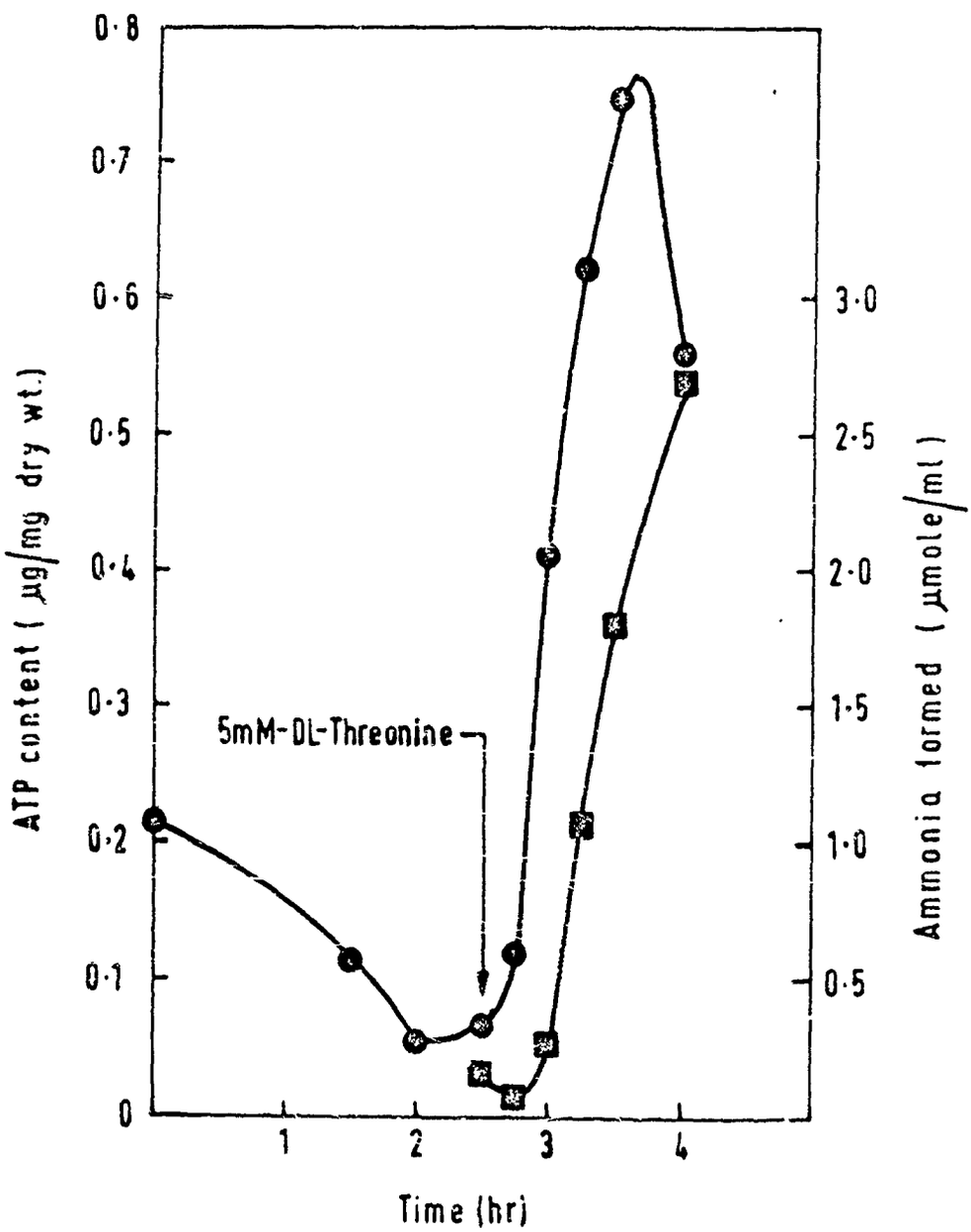


Fig. 15. Effect of pyruvate on the ATP content of starved Peptococcus prévotii.

Cell density, 1.45 mg./ml.. Pyruvate to a final concentration of 5 mM was added after 2 hr. 45 min. starvation. ATP content, ●; pyruvate concentration, ■.

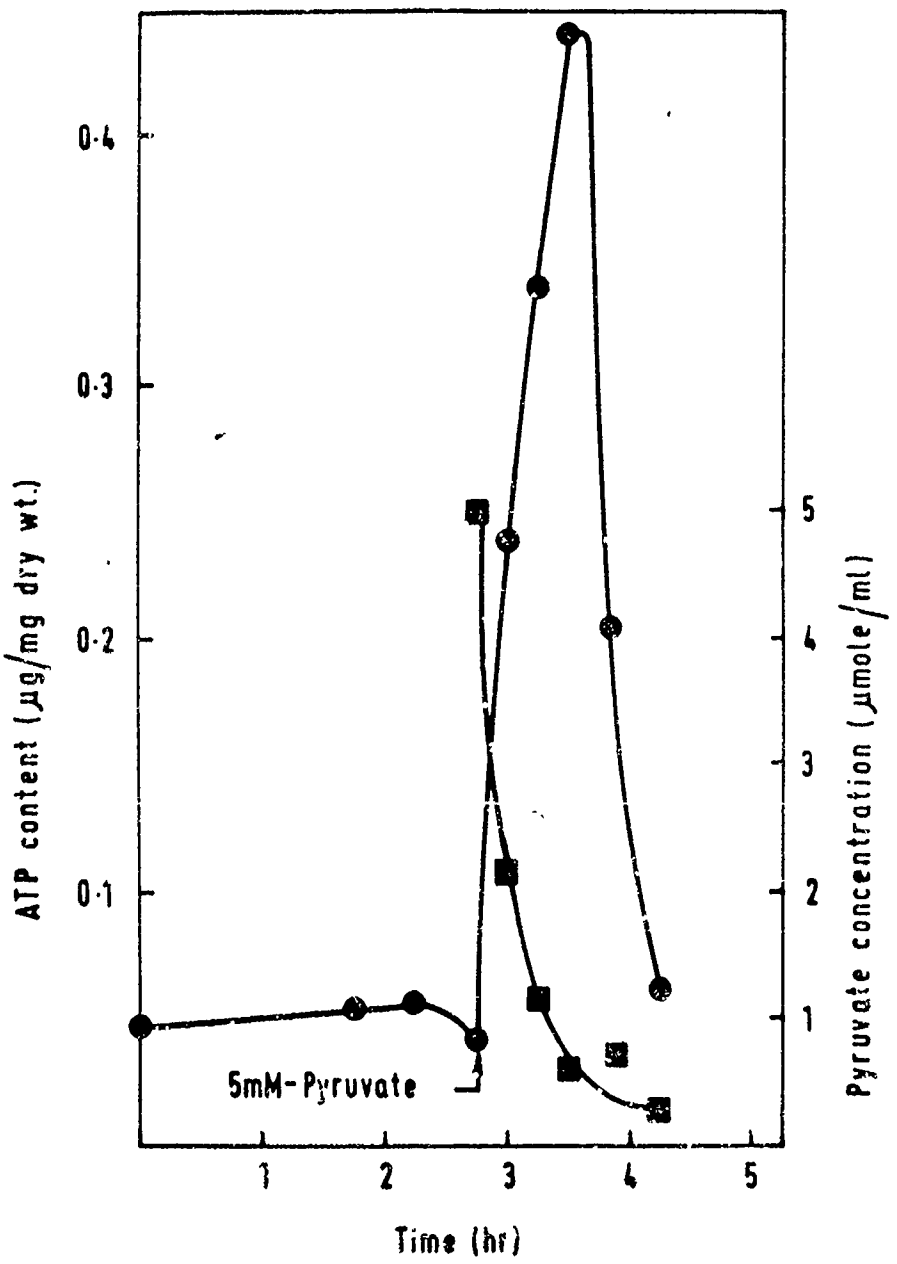


Fig. 16. Effect of 2-oxobutyrate on the ATP content of starved Peptococcus prévotii.

Cell density, 0.95 mg./ml.. 2-Oxobutyrate was added to a final concentration of 5.4 mM after 3 hr. starvation. ATP content, ●; 2-oxobutyrate concentration, ■.

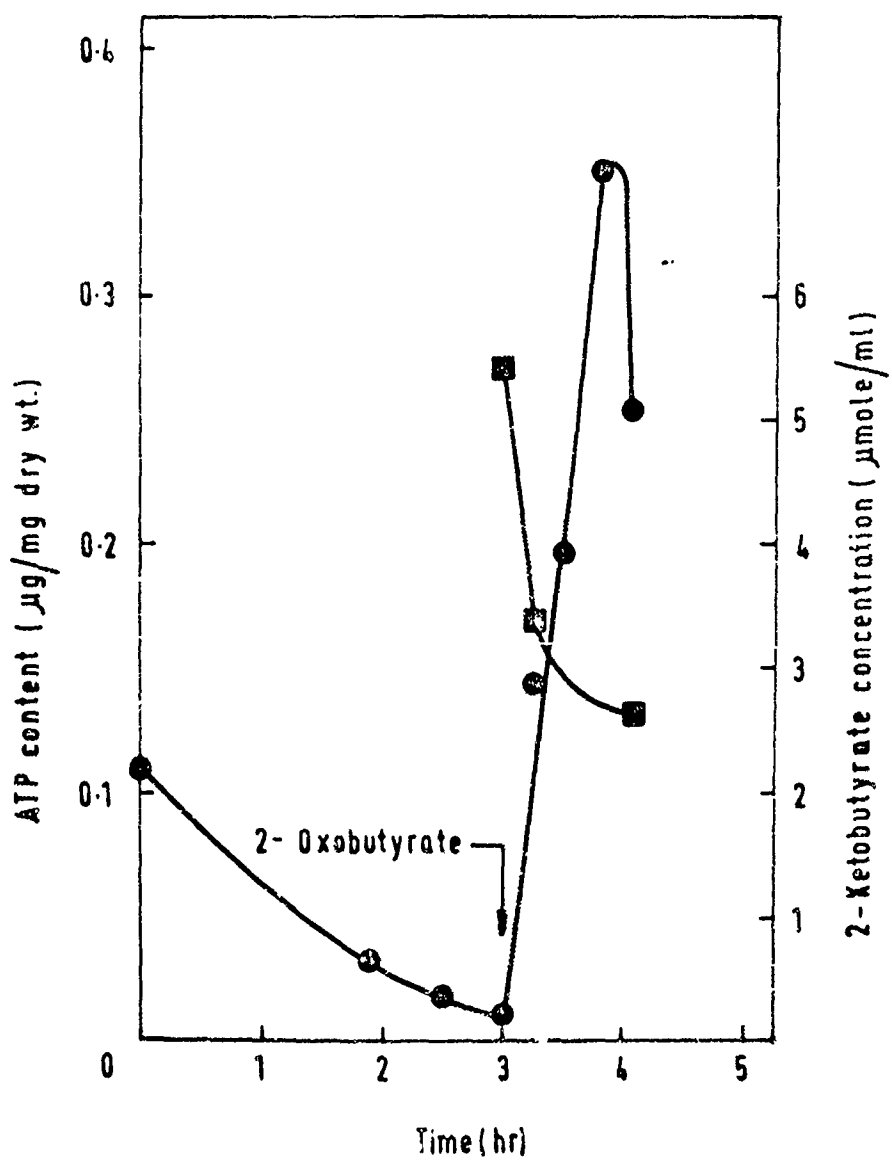
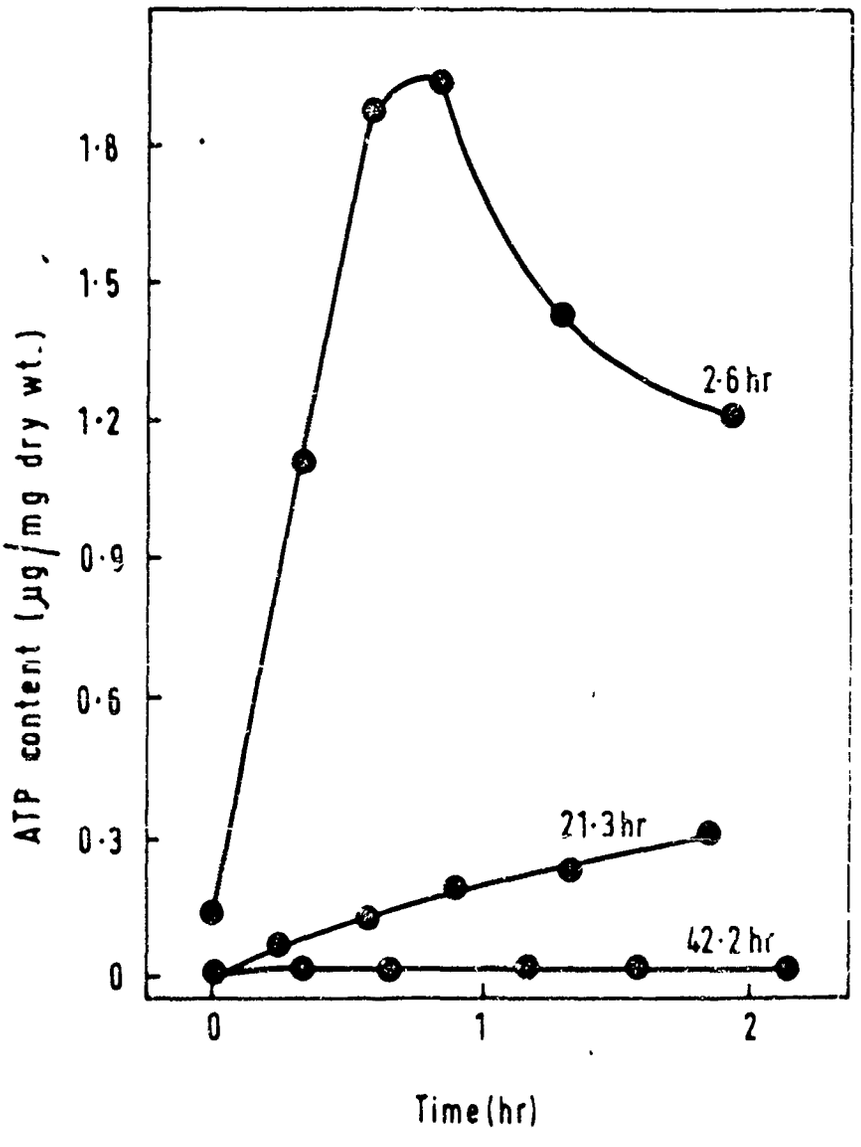


Fig. 17. Effect of L-serine on the ATP content of starved Peptococcus prévotii during prolonged starvation.

Cell density, 0.72 mg./ml.. L-Serine was added to suitable samples of suspension after starvation for 2 hr. 40 min., 21 hr. 20 min. and 42 hr. 10 min., to give final concentrations of 10 mM.



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13. ABSTRACT The continuation of an investigation of the endogenous metabolism and survival of non-spore-forming anaerobic bacteria under conditions of starvation is reported. The two glucose-fermenting organisms of the genus Zymomonas, Zymomonas anaerobia and Zymomonas mobilis, differ in that RNA is lost very much more rapidly from Z. mobilis than from Z. anaerobia, although the survival patterns are very similar. Both organisms are resistant to prolonged starvation. Magnesium ions suppress RNA degradation but do not confer protection against death. The remarkably constant protein content of Z. anaerobia does not seem to mask any kind of turnover or preferential degradation and resynthesis of specific proteins. The protein of Peptococcus prevotii is also remarkably stable and the observed release of ammonia is probably derived from deamination of nucleotide bases. The endogenous metabolism of this organism continues in the presence of exogenous glucose. To study the energetics of starvation the ATP contents of Z. anaerobia and P. prevotii have been measured by the firefly luciferase technique. In both cases the ATP content falls rapidly within the initial few hours and then levels out at a small value; it is clearly not related directly to the viability. The capacity of P. prevotii to synthesize ATP during starvation was therefore tested at intervals by adding pulses of suitable energy-yielding substrates such as serine, threonine and pyruvate. The ability to produce ATP on addition of serine is virtually abolished after 42 hours of starvation.

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